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ORIGINAL ARTICLE

IL-6 significantly correlates with p-STAT3 expression and presents high variceal bleeding with mortality in cirrhotic patients: A cross-sectional study

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KEYWORDS
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p-STAT3

Abstract *Background/Purpose:* Effective mediators activate downstream transducers regulating inflammation and angiogenesis. Correlation among mediators IL-6, IL-27, TNF-α, and VEGF with STAT proteins at diverse clinical-pathologic stages of cirrhotic patients remains limited.
Methods: Plasma mediators were assayed from 158 naïve liver cirrhosis (LC-total group) and 144 non-LC individuals. The LC-total group included 69 hepatitis B virus-infected (LC-HBV) patients, 40 hepatitis C virus-infected (LC-HCV) patients, and 49 patients without HBV−/HCV− infection (LC-NBNC). Another 144 non-LC individuals comprised 54 healthy persons (HG) and 90 chronic hepatitis patients (CH-total) as the control group. To correlate with plasma mediators, 52 paired liver tissues (CH: 41 and LC: 11 cases) served for p-STAT1 and p-STAT3 immunostaining.

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Results: Although IL-6, IL-27, TNF-α, and VEGF were expressed significantly in CH-total versus HG ($p = 0.011$, $p < 0.001$, $p = 0.007$, $p = 0.004$, respectively) and overall viral hepatitis patients versus HG ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, respectively), only IL-6 presented the strongest correlation in cirrhotic patients than noncirrhotic patients (LC-HBV vs. HG, $p < 0.001$, vs. CH-HBV, $p = 0.001$; LC-HCV vs. HG, $p = 0.001$, vs. CH-HCV, $p = 0.031$; LC-NBNC vs. HG, $p < 0.001$). Over-expressed IL-6 linked with poorer liver function (albumin: $r = -0.346$, $p < 0.001$; bilirubin: $r = 0.271$, $p = 0.001$; INR: $r = 0.308$, $p < 0.001$; Child-Turcotte-Pugh Classification C vs. A or B, $p = 0.001$, $p = 0.007$, respectively), variceal severity ($p = 0.045$), and bleeding ($p = 0.047$), as well as patients' mortality ($p = 0.005$). Furthermore, plasma IL-6 significantly correlated with tissues p-STAT3 expression ($r = 0.737$, $p = 0.010$) (IL-27: $r = 0.078$, $p = 0.820$; TNF-α: $r = -0.145$, $p = 0.670$; VEGF: $r = 0.142$, $p = 0.678$) in cirrhotic patients than noncirrhotic patients.
Conclusion: Over-expression of IL-6 reflects hepatic dysfunction and varices bleeding with mortality, as well as correlates p-STAT3 expression in cirrhotic patients.

Introduction

Hepatitis B virus (HBV), hepatitis C virus (HCV) infections, and chronic alcohol abuse all contribute to three important etiologies of liver cirrhosis (LC) worldwide,[1,2] while deteriorating hepatic preservation in cirrhotic patients, varices formation and rupture, among the complications, is frequently a leading cause of death.[3] Prone to variceal rupture, resistance to the blood flow of the portal vein gradually increases and induces hypertensive situation in the portal venous system, which develops into portal hypertension and results in the engorgement of the portal—systemic collateral vessels. Finally, these engorged vessels can lead to the development of varices at various gastrointestinal locations.[4–6] Although the invasive modalities such as esophagogastroduodenoscopy and hepatic venous pressure gradient monitoring were introduced,[7–9] they are limited in these high-risk populations owing to being invasive procedures that could result in further vessel or hepatic injury in clinical scenarios. Therefore, finding noninvasive optimal biomarkers which would allow detection before varices formation or rupture and lead to a further decrease in mortality of cirrhotic patients has become a critical issue.

Among well-recognized mediators, biological activities by vascular endothelial growth factor (VEGF), interleukin (IL)-27, IL-6, and tumor necrosis factor-alpha (TNF-α) have been implicated in regulating inflammation and/or angiogenesis in liver diseases. VEGF can induce development of portosystemic collateral vessels in animal studies.[10–14] IL-27 not only can act on hepatocytes against viral activity, but also can suppress tumor proliferation.[15,16] IL-6 might reflect more active hepatic necroinflammation and be associated with the severity of disease.[17–21] TNF-α is not only a proinflammatory cytokine,[22,23] but also can regulate the development and rupture of esophageal varices in animal studies.[24-29] However, the biological functions are mediated by various types of signaling pathways. The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway has been observed, which leads to cell proliferation, protection from apoptosis, and increased metastatic potential in liver disease.[21] However, the interrelations of biological effects among these mediators with STATs proteins remain limited in the studies of cell lines and animal models.[10–12,27–29]

To elucidate the clinical importance of IL-6, IL-27, TNF-α, and VEGF in varices development with rupture and mortality among cirrhotic patients, as well as their correlation with STAT1 and STAT3 proteins, we conducted this study in different clinical-pathologic stages of liver diseases. Findings on biological mechanisms of these molecules and their interrelations with varices and mortality might increase our understanding to create new therapeutic modalities for managing liver cirrhosis.

Methods

Setting and participants

After informed consent and exclusion of patients who did not meeting the enrollment criteria, 302 patients with a well-characterized clinical condition for plasma mediators assay, including 158 cirrhotic patients without previous variceal hemorrhage (LC-total group), 90 hepatitis patients (CH-total group) and 54 healthy individuals (HG) were enrolled at China Medical University Hospital in Taichung, Taiwan. The period of patient enrollment was from January 2010 to March 2012 and their clinical outcomes were followed until January 2014 to evaluate cirrhotic patients at 3-month, 6-month, and final mortality stages. As per clinical serological diagnoses, the LC-total group was categorized into three subgroups, including 69 patients with positive HBsAg for longer than 6 months (LC-HBV group), 40 patients with positive anti-HCV Ab for longer than 6 months (LC-HCV group), and 49 patients with negative HBsAg and anti—anti-HCV markers (LC-NBNC group). CH-total group comprised those without LC but who are positive for HBsAg or the anti-HCV marker for longer than 6 months, including 25 HBV-infected and 65 HCV-infected patients. The HG group included those without LC and negative for HBsAg/anti-HCV marker. Another 52 paired liver tissues, including 11 LC

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cases (8 HBV cases and 3 HCV cases) and 41 CH cases (10 HBV cases and 31 HCV cases) were obtained for immunostaining of STAT1 phosphorylation (p-STAT1) and STAT3 phosphorylation (p-STAT3).

The definition and severity of LC were based on (1) abdomen ultrasonography finding combined clinical signs with biochemical examinations, including shrunken liver size, splenomegaly, jaundice, ascites, hyperbilirubinemia, reversed levels of serum aspartate transaminase (AST) and alanine Transaminase (ALT), prolonged serum prothrombin time, and the albumin/globulin (A/G) ratio <1 etc.; or (2) liver biopsy and histopathology showing a METAVIR score of 4. Further classifications of disease severity were according to the Child—Turcotte—Pugh (CTP) Classification rather than the model for end-stage liver disease (MELD) score, which was used to predict the outcome of cirrhotic patients in general.[30] In addition, the severity levels of esophageal varices were determined according to esophagogastroduodenoscopy findings and classified from Grade I (minimally elevated veins above the esophageal mucosal surface) to Grade III (occupied more than one-third of the esophageal lumen). The variceal rupture meant patients accepted esophagogastroduodenoscopy intervention, such as sclerotherapy or band ligation, owing to the finding of (1) active bleeding from a varix; or (2) white nipple or clots overlying varix combined bleeding evidence before the emergency room, with low hemoglobin data. They were all admitted for further medical treatment. The definition of 3-month (early) and 6-month (chronic) mortality meant the cirrhotic patient died owing to disease progression since enrollment during the study period.

The LC or CH patients who had (1) coinfection or superinfection with hepatitis A virus (HAV), HBV, HCV, or human immunodeficiency virus (HIV); (2) previous antiviral agents, i.e. interferon, nucleotide agents, or immunomodulatory agents; (3) autoimmune hepatitis; or (4) previous variceal hemorrhage and received ligation or sclerotherapy, were excluded from this study. All procedures were followed in accordance with the ethical standards of the responsible Committee on Human Experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. The Institutional Review Board of China Medical University Hospital, Taichung, Taiwan, also approved this study.

Serological virus markers and liver biochemical assays methodology

Serum HBV markers, anti-HCV antibodies, HBV DNA, and HCV RNA levels were assessed by commercial enzyme immunoassay (AxSYM, Abbott, North Chicago, IL, USA; Abbott HCV EIA 2.0; Abbott Laboratories; Cobas Amplicor HCV Monitor 2.0; Roche Diagnostics, Branchburg, NJ, USA). Serum albumin, AST, and ALT, AFP, bilirubin, coagulation tests, and creatinine were determined using an autoanalyzer (TBA-30FR, Toshiba, Tokyo, Japan).

Estimation of plasma mediators and tissue immunohistochemistry

Venous plasma samples obtained from peripheral vein of all enrolled cases (for cases with variceal rupture, sample collected after the diagnosis by EGD within 2 days) and immediately centrifuged and stored at -80°C. Quantification of IL-6, IL-27, TNF-α and VEGF by specific enzyme-linked immunosorbant assay was made using commercially available kits within 2 weeks (kits for IL-6, IL-27, and TNF-α were from eBioscience, San Diego, CA; the VEGF kit was from Antigenix American, Huntington Station, NY). Results were expressed in picograms per milliliter (pg/mL); liver tissues were fixed in 10% formalin and embedded in paraffin. Blocks were sectioned at 4 μm for each tissue and three pieces of each specimen stained, including one without and two with phosphorylation according to a standard protocol (Cell Signaling Technology, Inc. 3 Trask Lane, Danvers, MA, USA). As a result, immunostaining of p-STAT1 and p-STAT3 mean active form was quantified by counting positively stained cytoplasma and nuclei of hepatocytes per 10 high-power fields ($\times$400 magnifications) microscopically from each specimen, respectively. Positive immunostain was considered when $\geqq$10% nuclei or cytoplasma of hepatocytes were stained. The immunoreactivity expression was categorized as Level I (mean $<$10% nuclei or cytoplasma of hepatocytes stained), II (mean $\geqq$10% to $<$25% nuclei or cytoplasma of hepatocytes stained), or III (mean$\geqq$25% nuclei or cytoplasma of hepatocytes stained).

Statistical analysis

The clinical parameters in Table 1 were expressed as mean $\pm$ standard deviation. Cytokine expression was presented as mean $\pm$ standard error mean and each group of experiments was repeated at least twice to confirm the data. Continuous variables were assessed using the Student *t*-test and Pearson correlation, with data analyzed using SPSS version 17.0 for Microsoft Windows (SPSS, Chicago, IL, USA). A two-sided p value <0.05 indicated statistical significance.

Results

Patients' demographic and clinical characteristics

Table 1 shows the baseline characteristics of the three major groups. Patients in the LC-total group were older than those in the CH-total group and HG (55.55 $\pm$ 13.14 vs. 50.13 $\pm$ 14.36 vs. 42.87 $\pm$ 13.35 years of age, respectively), which was compatible with the distribution of liver diseases.

IL-6 presented the strongest expression in cirrhotic patients than noncirrhotic patients

In HBV- or HCV-infected liver diseases, four mediators were significantly expressed in the CH-total group versus HG (2.76 $\pm$ 0.88 vs. 0.46 $\pm$ 0.10 pg/mL in IL-6, $p = 0.011$; 165.86 $\pm$ 33.68 vs. 9.83 $\pm$ 3.26 pg/mL in IL-27, $p < 0.001$; 67.10 $\pm$ 23.53 vs. 1.76 $\pm$ 1.49 pg/mL in TNF-α, $p = 0.007$; 4.81 $\pm$ 1.48 vs. 0.40 $\pm$ 0.07 pg/mL in VEGF, $p = 0.004$, respectively) (Fig. 1) and overall viral hepatitis patients (CH-total and LC-HBV and LC-HCV) versus HG (8.90 $\pm$ 1.44 vs. 0.46 $\pm$ 0.10 pg/mL in IL-6, $p < 0.001$; 188.89 $\pm$ 36.21 vs. 9.83 $\pm$ 3.26 pg/mL in IL-27, $p < 0.001$; 56.54 $\pm$ 14.53 vs.

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Table 1 Baseline characteristics of all enrolled cases ($N = 302$)

Demographics	Healthy group ($n = 54$)	CH group ($n = 90$)		Cirrhotic group ($n = 158$)		
		CH-HBV ($n = 25$)	CH-HCV ($n = 65$)	LC-NBNC ($n = 49$)	LC-HBV ($n = 69$)	LC-HCV ($n = 40$)
Age (y) (range)	42.87 ± 13.35 (19–68)	44.2 ± 12.41 (19–76)	52.42 ± 14.48 (19–74)	53.04 ± 12.86 (29–81)	53.28 ± 11.36 (32–82)	62.55 ± 14.13 (35–89)
Sex (Male) (%)	33 (61.1%)	18 (72%)	33 (50.8%)	41 (83.7%)	57 (82.6%)	18 (45.0%)
Varices/variceal bleeding (n/n)	0/0	0/0	0/0	37/9	34/13	21/10
Child-Pugh score (stage A/B/C)	−/−/−	−/−/−	−/−/−	18/11/20	32/16/21	11/11/18
Biochemical values						
Albumin (g/dL)	4.44 ± 0.37 (3.5–5.0)	4.27 ± 0.45 (3.4–5.1)	4.33 ± 0.38 (3.3–5.1)	3.29 ± 0.73 (1.4–4.5)	3.45 ± 0.71 (2.0–4.6)	3.05 ± 0.76 (1.6–4.8)
Bilirubin (mg/dL)	0.8 ± 0.23 (0.25–1.30)	0.95 ± 0.36 (0.52–2.23)	1.0 ± 0.52 (0.42–4.11)	4.49 ± 5.92 (0.32–26.49)	5.80 ± 9.87 (0.45–38.82)	3.12 ± 4.77 (0.17–23.41)
Creatinine (mg/dL)	0.83 ± 0.20 (0.49–1.51)	0.8 ± 0.15 (0.59–1.03)	0.83 ± 0.24 (0.43–1.53)	1.14 ± 0.73 (0.42–4.36)	1.34 ± 1.44 (0.52–8.75)	1.51 ± 1.85 (0.35–11.21)
AST (IU/L)	21.86 ± 6.28 (14–51)	50.6 ± 43.53 (12–201)	73.56 ± 79.46 (17–463)	58.31 ± 35.25 (11–147)	146.06 ± 332.62 (21–2098)	70.31 ± 40.16 (23–192)
ALT (IU/L)	20.93 ± 7.16 (10–40)	65.35 ± 53.69 (16–218)	95.55 ± 166.47 (13–1330)	30.24 ± 18.17 (11–117)	150.97 ± 524.80 (11–4200)	45.68 ± 28.26 (12–109)
INR	0.96 ± 0.06 (0.86–1.18)	1.02 ± 0.07 (0.87–1.15)	1.0 ± 0.06 (0.89–1.19)	1.39 ± 0.41 (0.89–2.58)	1.47 ± 0.70 (0.89–3.93)	1.32 ± 0.36 (0.92–2.57)
Platelet ($10^3/\mu L$)	245.46 ± 57.22 (138–351)	191.11 ± 47.73 (101–283)	188.95 ± 52.67 (92–378)	95.6 ± 54.41 (23–252)	102.73 ± 61.98 (26–387)	97.75 ± 47.24 (33–214)
AFP	2.78 ± 1.45 (1.0–8.02)	15.33 ± 28.76 (0.99–104.24)	14.58 ± 75.28 (1.21–611.11)	8.78 ± 12.64 (1.24–76.72)	28.22 ± 81.67 (1.24–570.18)	9.21 ± 10.6 (1.66–64.93)
Virological values						
HBeAg (+) (%)	0	11 (44%)	0	0	9 (13.0%)	0
Viral loads (copies/mL)	0	$6.95 \times 10^8 \pm 2.59 \times 10^9$ (<34.92–1.27×10^{10})	$8.95 \times 10^6 \pm 1.95 \times 10^7$ (<40.5–1.33×10^8)	0	$2.67 \times 10^7 \pm 1.15 \times 10^8$ (<34.92–6.40×10^8)	$4.70 \times 10^6 \pm 7.82 \times 10^6$ (<40.5–2.01×10^7)

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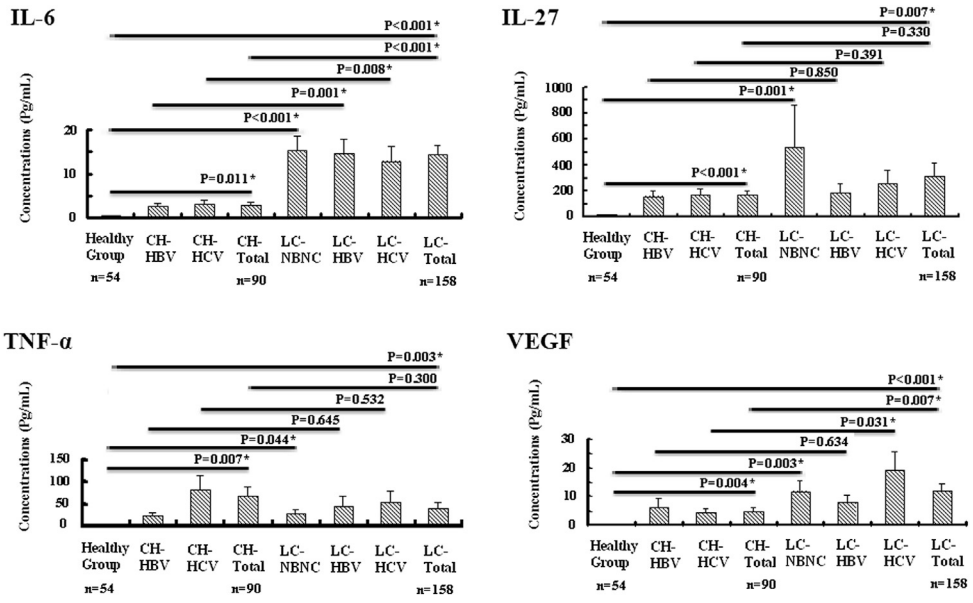

Figure 1. The expression of IL-6, IL-27, TNF-α, and VEGF in separate subgroups. * A value of $p < 0.05$ was defined as statistically significant.

1.76 ± 1.49 pg/mL in TNF-α, $p < 0.001$; 8.86 ± 1.67 vs. 0.40 ± 0.07 pg/mL in VEGF, $p < 0.001$, respectively).

Whereas chronic hepatitis progressed to cirrhosis, both IL-6 and VEGF rather than IL-27 with TNF-α profiles were expressed predominantly in the LC-total group versus HG (14.40 ± 1.96 vs. 0.46 ± 0.10 pg/mL in IL-6, $p < 0.001$; 309.49 ± 109.22 vs. 9.83 ± 3.26 pg/mL in IL-27, $p = 0.007$; 41.35 ± 13.07 vs. 1.76 ± 1.49 pg/mL in TNF-α, $p = 0.003$; 12.09 ± 2.21 vs. 0.40 ± 0.07 pg/mL in VEGF, $p < 0.001$, respectively) and LC-total versus CH-total groups (14.40 ± 1.96 vs. 2.76 ± 0.88 pg/mL in IL-6, $p < 0.001$; 12.09 ± 2.21 vs. 4.81 ± 1.48 pg/mL in VEGF, $p = 0.007$, respectively).

However, IL-6 among four mediators was over-expressed in separate LC groups: LC-HBV group versus HG (14.63 ± 3.32 vs. 0.46 ± 0.10 pg/mL in IL-6, $p < 0.001$; 179.99 ± 72.22 vs. 9.83 ± 3.26 pg/mL in IL-27, $p = 0.021$; 43.74 ± 25.43 vs. 1.76 ± 1.49 pg/mL in TNF-α, $p < 0.001$; 8.17 ± 2.09 vs. 0.40 ± 0.07 pg/mL in VEGF, $p < 0.001$) and CH-HBV group (14.63 ± 3.32 vs. 2.46 ± 0.75 pg/mL in IL-6, $p = 0.001$); LC-HCV group vs. HG (12.81 ± 3.36 vs. 0.46 ± 0.10 pg/mL in IL-6, $p = 0.001$; 256.09 ± 107.02 vs. 9.83 ± 3.26 pg/mL in IL-27, $p = 0.027$; 55.16 ± 23.29 vs. 1.76 ± 1.49 pg/mL, $p < 0.001$ in TNF-α; 19.16 ± 6.49 vs. 0.40 ± 0.07 pg/mL in VEGF, $p = 0.006$) and CH-HCV group (12.81 ± 3.36 vs. 2.88 ± 1.18 pg/mL, $p = 0.008$ in IL-6; 19.16 ± 6.49 vs. 4.26 ± 1.60 pg/mL in VEGF, $p = 0.031$); and LC-NBNC group vs. HG (15.36 ± 3.28 vs. 0.46 ± 0.10 pg/mL in IL-6, $p < 0.001$; 535.45 ± 325.90 vs. 9.83 ± 3.26 pg/mL in IL-27, $p = 0.001$; 26.73 ± 11.96 vs. 1.76 ± 1.49 pg/mL in TNF-α, $p = 0.044$; 11.83 ± 3.66 vs. 0.40 ± 0.07 pg/mL in VEGF, $p = 0.003$) (Fig. 1).

IL-6 presented the strongest correlation with fibrosis severity in biopsy patients

For correlation between fibrosis scores of tissues with serum mediator expressions in 52 biopsy cases (Score 1 = 20 cases, Score 2 = 13 cases, Score 3 = 8 cases, Score 4 = 11 cases), only IL-6 showed a positive correlation with fibrosis severity ($r = 0.313$, $p = 0.024$).

Over-expression of IL-6 significantly correlated with deteriorating liver condition in cirrhotic patients

Among four mediators, IL-6 overexpression not only presented the strongest correlation with clinical factors associated with liver function, such as albumin ($r = -0.346$; $p < 0.001$), bilirubin ($r = 0.271$; $p = 0.001$), and international normalized ratio (INR) levels ($r = 0.308$; $p < 0.001$) (Table 2) but also with liver preservation according to the CTP classification (24.08 ± 3.92 vs. 8.06 ± 2.1 pg/mL, $p = 0.001$ in Class C vs. A; 24.08 ± 3.92 vs. 9.55 ± 3.51 pg/mL, $p = 0.007$ in Class C vs. B). By contrast, IL-27, TNF-α, and VEGF could not show significant statistical difference in each group (Fig. 2).

Raised IL-6 expression presented significant correlation with varices formation, severity, and rupture in cirrhotic patients

Although IL-27 and TNF-α same IL-6 showed significant expression in presence ($n = 92$ cases) versus not ($n = 66$ cases) of varices in all cirrhotic patients (16.47 ± 2.65 vs. 11.52 ± 2.86 pg/mL in IL-6, $p = 0.045$; 413.60 ± 179.01 vs. 164.36 ± 76.73 pg/mL in IL-27, $p = 0.027$; 63.18 ± 21.92 vs. 10.94 ± 5.01 pg/mL in TNF-α, $p = 0.022$, respectively) (Fig. 3A), both IL-27 and TNF-α could not significantly activate in presence ($n = 32$ cases) versus not ($n = 60$ cases) of variceal bleeding among patients with varices. By contrast, only IL-6 expression significantly correlated with variceal bleeding (22.21 ± 4.92 vs. 13.41 ± 3.06 pg/mL, $p = 0.047$) (Fig. 3B) and severity of varices (Grade 0/1/2/

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Table 2 Correlations between IL-6, IL-27, TNF-α, and VEGF with clinical biochemical data in naïve cirrhotic patients ($N = 158$)

	IL-6		IL-27		TNF-α		VEGF	
	γ	*p*	γ	*p*	γ	*p*	γ	*p*
Age (y)	−0.075	0.349	−0.131	0.101	−0.016	0.846	0.081	0.309
Sex (male/female)	−0.036	0.653	−0.171	0.032*	−0.093	0.247	−0.263	0.001*
Albumin (g/dL)	−0.346	<0.001*	−0.089	0.118	0.701	0.154	−0.171	0.039*
Bilirubin (mg/dL)	0.271	0.001*	0.064	0.422	0.012	0.879	−0.034	0.672
Creatinine (mg/dL)	0.013	0.875	−0.059	0.474	−0.054	0.510	−0.028	0.732
AST (IU/L)	0.101	0.226	0.003	0.968	−0.045	0.591	−0.031	0.709
ALT (IU/L)	0.115	0.151	−0.027	0.735	−0.031	0.701	−0.039	0.626
INR	0.308	<0.001*	0.096	0.240	0.067	0.415	0.017	0.835
Platelet ($10^3/\mu L$)	−0.062	0.448	0.054	0.514	−0.127	0.119	−0.091	0.265
AFP (ng/mL)	−0.057	0.484	−0.022	0.787	−0.005	0.949	0.053	0.514
HBV or HCV (positive)	0.026	0.742	0.111	0.166	−0.060	0.455	−0.006	0.939

*A value of $p < 0.05$ was defined as significant.

3 = 66/38/40/14 cases) (Grade 0 vs. 1 vs. 2 vs. 3 in IL-6 expression: 11.57 ± 2.98 vs. 15.30 ± 3.74 vs. 15.08 ± 3.68 vs. 22.09 ± 8.63 pg/mL, respectively, $r = 0.190$ and $p = 0.017$; IL-27 expression: $r = 0.084$ and $p = 0.294$; TNF-α expression: $r = 0.126$ and $p = 0.113$; VEGF expression: $r = 0.070$ and $p = 0.383$, respectively).

Raised IL-6 expression presented the strongest correlation with mortality in cirrhotic patients

Compatible with the above findings, elevated IL-6 levels significantly correlated with mortality including 3-month or 6-month survival (≤3-month vs. >3-month survival: 31.03 ± 7.24 vs. 12.65 ± 1.98 pg/mL, $p = 0.006$; ≤6-month vs. >6-month survival: 30.66 ± 6.24 vs. 12.04 ± 1.98 pg/mL, $p = 0.009$, respectively) (Fig. 4A and B), and also presented an important factor to predict 24-month mortality in cirrhotic patients ($p = 0.005$) such as variceal bleeding ($p = 0.034$) and lower albumin levels ($p = 0.008$) (Table 3). By contrast, elevated IL-27 and TNF-α and VEGF levels showed insignificant correlation with mortality (Fig. 4A and B, Table 3). Furthermore, IL-6 presented better sensitivity (65.35%), specificity (71.93%), negative predictive values (NPV) (53.95%), and positive predictive values (PPV) (80.45%) to predict mortality than other cytokines (53.02%, 66.67%, 7.89%, and 96.34% in IL-27; 57.89%, 57.14%, 47.37%, and 67.07% in TNF-α; 55.32%, 76.47%, 17.11%, and 95.12% in VEGF, respectively).

Tissue p-STAT3 showed a higher immunostaining rate than p-STAT1 and correlated with plasma IL-6 expression in biopsy patients

To explore the clinical relationship between tissues p-STAT proteins and plasma four mediators, we examined the immunoreactivities expression of p-STAT1 and p-STAT3 in 52 biopsy patients. As shown in Fig. 5A, we found p-STAT3 presented a higher immunostaining rate (≧10% immunostaining of hepatocytes) than p-STAT1 in all liver

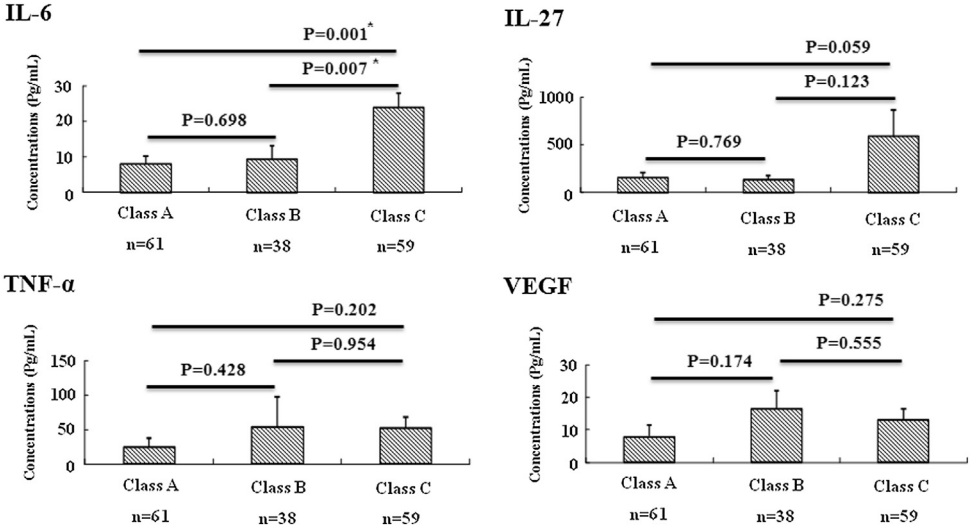

Figure 2. IL-6 expression presented significant correlation with deteriorating liver condition in cirrhotic patients. * A value of $p < 0.05$ was defined as statistically significant.

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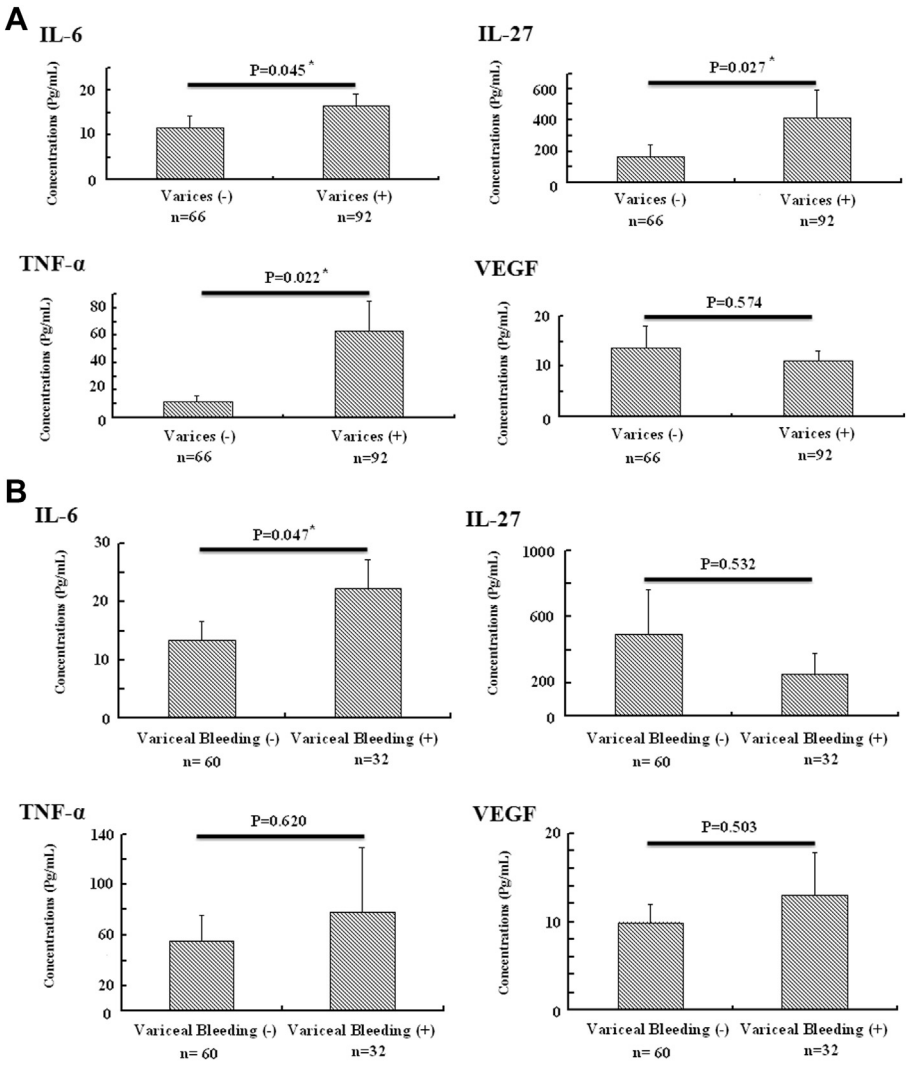

Figure 3. IL-6 expression presented significant correlation with varices formation (A) and variceal bleeding (B) in cirrhotic patients. * A value of $p < 0.05$ was defined as statistically significant.

specimens (32/52 vs. 19/52, $p = 0.003$). Moreover, increasing immunoreactivities levels of p-STAT3 rather than p-STAT1 also significantly correlated with IL-6 expression in all LC with CH patients (p-STAT3 vs. IL-6: $r = 0.316$, $p = 0.022$; vs. IL-27: $r = 0.116$, $p = 0.239$; vs. TNF-α: $r = 0.228$, $p = 0.105$; vs. VEGF: $r = 0.329$, $p = 0.017$, respectively); (p-STAT1 vs. IL-6: $r = -0.137$, $p = 0.334$; vs. IL-27: $r = 0.000$, $p = 0.998$; vs. TNF-α: $r = 0.129$, $p = 0.364$; vs. VEGF: $r = 0.094$, $p = 0.507$, respectively); and LC patients (p-STAT3 vs. IL-6: $r = 0.737$, $p = 0.010$; vs. IL-27: $r = 0.078$, $p = 0.820$; vs. TNF-α: $r = -0.145$, $p = 0.670$; vs. VEGF: $r = 0.142$, $p = 0.678$, respectively); (p-STAT1 vs. IL-6: $r = -0.400$, $p = 0.223$; vs. IL-27: $r = -0.278$, $p = 0.408$; vs. TNF-α: $r = -0.420$, $p = 0.198$; vs. VEGF: $r = -0.493$, $p = 0.123$, respectively); (Fig. 5A and B).

Discussion

With deteriorating hepatic preservation, varices development with rupture becomes predominant and plays a leading cause of death among complications in cirrhotic patients.[3] Although modalities such as esophagogastroduodenoscopy and hepatic venous pressure gradient monitoring have been developed to investigate this fatal complication in clinical scenarios,[7–9] they are limited in these high-risk populations owing to being invasive procedures that could result in further vessel or hepatic injury. Therefore, finding noninvasive optimal biomarkers allowing early detection before varices formation or rupture to further decrease mortality of cirrhotic patients remains a critical issue.

In our study, IL-6, IL-27, TNF-α, and VEGF were significantly expressed in LC-total and CH-total groups than HG (Fig. 1), which was in accordance with previous studies where overexpression of cytokine or chemokine-mediated immunity could stimulate hepatocyte proliferation with fibrogenesis development in liver diseases.[10–21,27–29]

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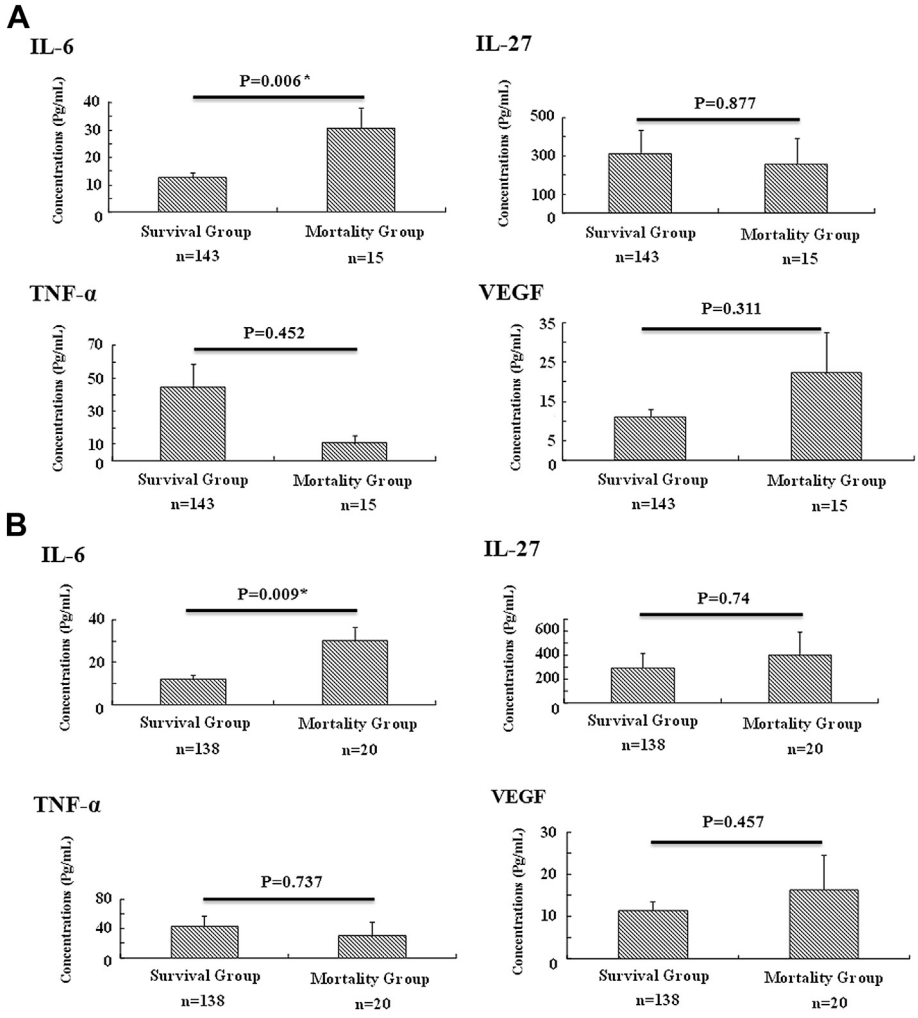

Figure 4. IL-6 presented the strongest correlation with 3-month (A) and 6-month mortality (B) in cirrhotic patients. * A value of $p < 0.05$ was defined as statistically significant.

Table 3 Univariate and multivariate analysis of mortality in cirrhotic patients ($N = 158$)

Variable	Risk ratio (95% CI)	p	Risk ratio (95% CI)	p
Demographics				
Age (>60 y)	1.665 (0.860–3.221)	0.130		
Gender (male)	1.027 (0.506–2.081)	0.942		
Varices (positive)	1.200 (0.637–2.262)	0.573		
Variceal bleeding (positive)	3.520 (1.508–8.218)	0.004*	3.955 (1.109–14.103)	0.034*
IL-6 (>7.10 pg/mL)	4.832 (2.380–9.812)	<0.001*	3.805 (1.482–9.766)	0.005*
IL-27 (>1247.5 pg/mL)	2.257 (0.544–9.364)	0.262		
TNF-α (>0.12 pg/mL)	1.833 (0.963–3.491)	0.065		
VEGF (>35.19 pg/mL)	4.024 (1.250–12.949)	0.020*		
AST (>34 IU/L)	2.184 (0.914–5.220)	0.079		
ALT (>40 IU/L)	0.824 (0.434–1.563)	0.552		
Albumin (>3.5 g/dL)	0.213 (0.102–0.447)	<0.001*	0.237 (0.082–0.684)	0.008*
Bilirubin (>2.0 mg/dL)	1.828 (0.966–3.459)	0.064		
Cr (>1.3 mg/dL)	1.912 (0.886–4.126)	0.099		
INR (>1.7)	2.379 (0.950–5.957)	0.064		
Platelet (>130 × 10^3/μL)	0.568 (0.271–1.192)	0.135		
AFP (>9 ng/mL)	1.007 (0.489–2.075)	0.985		

*A value of $p < 0.05$ was defined as significant.

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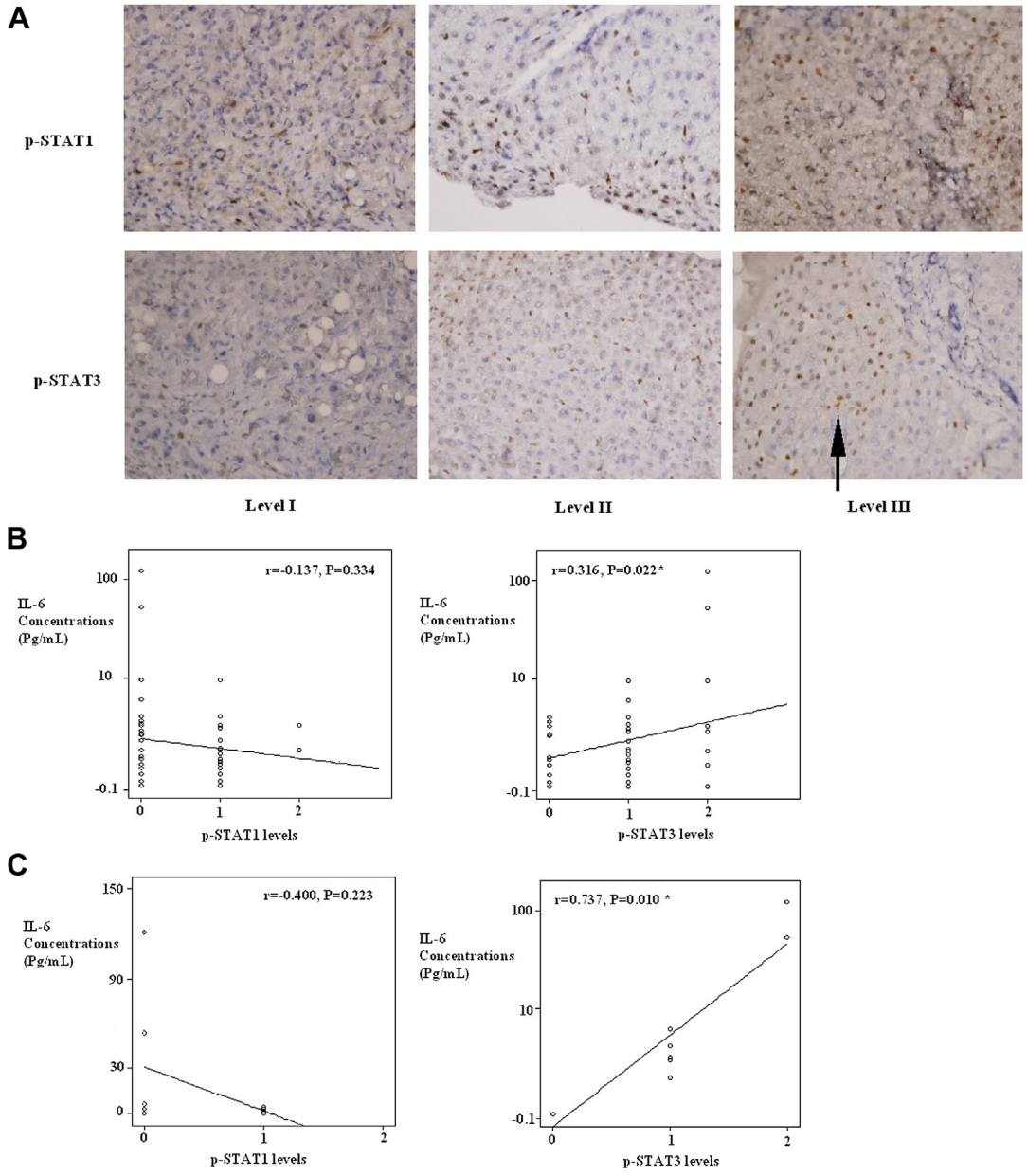

Figure 5. Immunohistochemical studies of p-STAT1 and p-STAT3 in three identical cirrhotic tissues. Immunoreactivity exhibited in nuclei or cytoplasm of hepatocyte was designated Level I (<10%), II (≧10% to <25%), or III (≧25%) [×400 magnification; the brown cell (arrow) shows positive immunostain] (A). Tissue p-STAT3 presented a positive correlation between increasing immunoreactivities levels with IL-6 expressions as compared with p-STAT1 with IL-6 in CH with LC patients ($r = 0.316$, $p = 0.022$; $r = -0.137$, $p = 0.334$, respectively) and in LC patients ($r = 0.737$, $p = 0.010$; $r = -0.400$, $p = 0.223$, respectively) (B,C). * A value of $p < 0.05$ was defined as statistically significant.

Nevertheless, we found upregulated IL-27, TNF-α, and VEGF levels could not reflect the liver condition under disease progression in cirrhotic patients. In turn, elevated IL-6 levels significantly correlated with deteriorating liver condition (Fig. 2) and three important clinical parameters evaluating condition severity, including albumin ($r = -0.346$; $p < 0.001$), bilirubin ($r = 0.271$; $p = 0.001$), and INR ($r = 0.308$; $p < 0.001$) (Table 2). These findings proved the action of IL-6 not only induced liver fibrogenesis like the other three mediators, but also upregulated the disease process to deteriorate hepatic preservation in cirrhotic patients.

With deterioration of liver preservation in cirrhotic patients, the risk of varices formation and severity could be increased and often threaten patients' survival owing to fatal complications.[3,31] In this study, we found TNF-α (as found in previous findings) was significantly expressed in patients with varices (Fig. 3A) and increased severity (Grade 0 vs. 1, $p = 0.017$)[22,23], but it did not correlate with increased variceal bleeding (Fig. 3B). By contrast, significant IL-6 expression not only reflected varices formation

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with severity (from Grade 0 to 3: 11.57 ± 2.98 vs. 15.30 ± 3.74 vs. 15.08 ± 3.68 vs. 22.09 ± 8.63 pg/mL, respectively, $r = 0.190$ and $p = 0.017$), but also increased bleeding risk (Fig. 3A and B). This proved the finding of prior studies where IL-6 was overexpressed in the hyperdynamic circulation and associated with variceal bleeding owing to increasing gastroduodenal/intestinal permeability in cirrhotic patients.[32,33] Increasing permeability would increase the risk of bacterial infection in bleeding patients, but our study found insignificant correlation between raised IL-6 expression and increased bacterial translocation in bleeding cases. However, higher IL-6 expression was found in cases with positive bacterial culture ($n = 21$) than those without bacterial culture ($n = 11$) (26.19 ± 5.31 vs. 14.60 ± 10.09 pg/mL, $p = 0.270$), which could be limited by cases numbers. In clinical scenarios, hepatic preservation frequently decides patient's outcome. In this study, over-expression of IL-6 also increased 3-month (Fig. 4A), 6-month (Fig. 4B), and 24-month mortality (Table 3). Its importance was equal or probably high for some important clinical features, such as variceal bleeding, lower albumin, higher bilirubin, and prolonged INR levels (Table 3). These findings identified upregulation of IL-6, which reflected more active hepatic severity,[17-21] development of varices with bleeding, as well as probably increased bacterial translocation through upregulation of permeability due to portal hypertension while deteriorating hepatic preservation in cirrhotic patients.[4–6,32,33]

Of the various downstream signaling targets of these mediators' profile, STAT and its family members, particularly in STAT1 and STAT3 proteins, played an important role in the pathogenesis of liver diseases.[21] However, the clinical relevance among these mediators with the STAT1 and STAT3 proteins remains not well understood and needs to be further elucidated in patients with different clinical stages of liver diseases. As for the resulting findings, only IL-6 significantly correlated with fibrosis severity in 52 biopsy patients ($r = 0.313$, $p = 0.024$). Also, we found p-STAT3 not only expressed a higher immunostaining rate than p-STAT1 (32/52 vs. 19/52, $p = 0.003$), but also its immunoreactivities levels were associated with IL-6 expression as compared with three mediators in all patients ($r = 0.116$, $p = 0.239$ in IL-27; $r = 0.228$, $p = 0.105$ in TNF-α; $r = 0.329$, $p = 0.017$ in VEGF) and particularly in LC patients ($r = 0.078$, $p = 0.820$; $r = -0.145$, $p = 0.670$; $r = 0.142$, $p = 0.678$, respectively), (Fig. 5B and C).

In the current study, we failed to demonstrate finding of all-participant liver tissues particularly in cirrhotic patients, and the limitation of cases numbers resulted in insignificant difference between p-STAT3 expression and the varices severity. However, this is limited based on ethical and safety considerations because patients with decompensation usually have high hemorrhagic risk. Additionally, β-blocker medication might benefit cirrhotic patients with variceal bleeding, but there was no significant difference between patients with or without β-blocker medication and bleeding (9/25 vs. 6/23, $p = 1.000$) as well as mortality (3-month: 3/25 vs. 9/67, $p = 1.000$; 6-month: 4/25 vs. 11/67, $p = 1.000$; time of study ending: 13/46 vs. 12/46, $p = 1.000$) in this study. Our study provided much useful information in liver diseases but was limited in a cross-sectional finding, which might have misleading considerations; therefore, further cohort studies need to be adopted in the future. The fluctuation of serum IL-6, IL-27, TNF-α, and VEGF concentrations in the host might be argued, but these molecules were examined from plasma in our study that were not affected by the amount of time between blood sampling and centrifugation according to a previous study.[34]

Based on these clinical findings in this study, we clearly demonstrated that plasma IL-6 not only plays the role of determined promoter in disease progression as well as varices development with severity, but also affects cirrhotic patient survival. The mechanism IL-6 might act on liver diseases through upregulation of p-STAT3 rather than p-STAT1 in the real world. We therefore believe through the generalizability of the study result that detection of plasma IL-6 expression before invasive procedures, such as esophagogastroduodenoscopy and hepatic venous pressure gradient monitoring, might provide clinicians with an effective reference allowing varices development and rupture to be earlier diagnosed.[35] Furthermore, it might create an attractive agent to prevent patient mortality in the future.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

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